5 *Botryomyces*

Dongyou Liu and R.R.M. Paterson

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5.1 INTRODUCTION

5.1.1 Classification and Morphology

The genus *Botryomyces* (Botrys, Greek for bunch of grapes, + mykes, Greek for fungus) is a meristematic black fungus belonging to the mitosporic Dothideomycetes group, class Dothideomycetes, subphylum Pezizomycotina, phylum *Ascomycota*, and kingdom Fungi [1]. The mitosporic Dothideomycetes group is divided into nine genera: *Asteromella*, *Botryomyces*, *Cenococcum*, *Cryomyces*, *Cyclothyrium*, *Cystocoleus*, *Racodium*, *Sclerostagonospora*, and *Seifertia*. The genus *Botryomyces* contains a single species *Botryomyces caespitosus*, which was first described from human skin lesions [2]; *Botryomyces angioformans* is a doubtful species from an unconfirmed disease of which no material has been preserved [3].

B. caespitosus colonies are pink when young and form restricted, meristematic cell clumps that disarticulate irregularly. Meristematic growth is characterized by the production of swollen isodiametric cells with thick cell walls, in which melanin is deposited. The fungus produces multicelled, irregularly septate, thick-walled spores, which may be regarded by some to be vegetative structures [2].

The genus *Botryomyces* is one of the 25 genera of meristematic black fungi that inhibit cracks in marble and rock surfaces in the Mediterranean Basin (Italy, Spain) and Ukraine. They are associated with biodeterioration of monuments, sculptures, and archaeological objects. Because meristematic black fungi present characteristics comparable to those of fungi isolated from deserts rocks, they are occasionally referred to as "microcolonial fungi." In addition, the term "black yeast" is sometimes used to describe black fungi that have yeast-like stages of reproduction and a meristematic growth pattern.

Meristematic black fungi are classified under four families within the *Ascomycota*: (i) *Herpotrichellaceae* (order Chaetothyriales) covers *Exophiala* and *Sarcynomices petricola*; (ii) *Dothideaceae* (order Dothideales) consists of some epiphytic species occasionally isolated from rocks such as *Trimmatostroma abietis*, *Aureobasidium pullulans*, and *Hortaea werneckii*; (iii) *Capnodiaceae* (order Capnodiales) includes *C. renispora*, which was isolated from a tile; and (iv) *Pleosporaceae* (order Pleosporales) includes *B. caespitosus*, which is closely related to *Alternaria* (which is also frequently found on stones) based on internal transcribed spacer (ITS) sequence analysis [4,5].

Stone-inhabiting meristematic black fungi tend to show intercrystalline growth by colonizing the weakest parts along marble crystals, leading to the detachments of crystals. They also grow preferentially in cavities and in already formed cracks and fissures, often producing a deepening of the fissures.

5.1.2 Clinical Features

Meristematic black fungi such as *B. caespitosus* are recognized agents of phaeohyphomycosis [6] as distinct from chromoblastomycosis. Readers should be aware of the term "botryomycosis," which, surprisingly, refers to a bacterial infection (affecting the skin, and sometimes the viscera due to *Staphylococcus aureus* and several other bacteria) [7] and should not be confused with the current disease. Phaeohyphomycosis is cosmopolitan although patients are usually adults and approximately half are immunocompromised; however, this figure has probably increased substantially until the present time. Lesions may occur on almost any part of the body, often on exposed areas, with the upper arm lesions being most prevalent. The most typical and common lesions are cutaneous or subcutaneous abscesses or cysts. Primary lesion is a single, discrete, asymptomatic small nodule and this evolves gradually to an encapsulated,

fluctuant abscess with a liquefied center. However, the overlying epidermis is hardly affected. Occasionally, a slightly elevated, granulomatous plaque appears when the main site of the lesion is in the dermis and epidermis. Infrequently, it is observed as a small verrucous nodule or a verrucous plaque comprising a coalescent nodule, which actually resembles chromoblastomycosis. Phaeohyphomycosis may involve the central nervous system or other internal organs (e.g., liver, lungs, and pancreas) and may appear as a hematogenous metastasis from cutaneous or subcutaneous infections or with no visible lesions.

Specifically, *B. caespitosus* may gain entry into human host by traumatic inoculation, through prolonged contact with domestic animals, and presumably via airborne dissemination of propagules. The fungus is responsible occasionally for chromoblastomycosis-like subcutaneous infections after trauma. Clinical symptoms may range from dermatomycosis (mycoses), cutaneous phaeohyphomycosis, mycotic granuloma—skin lesions on arms and legs, usually in immunocompromised patients or in patients with chronic renal failure, transplants, and immunosuppressive therapy [8,9]. Human infection of tonsils with *B. caespitosus* may exhibit recurrent tonsillitis, sore throat, dysphasia, high temperature, and enlarged tonsils. Upon examination, the tonsils may show "grains" in the crypts [10].

5.1.3 Diagnosis

As meristematic fungi lack pronounced diagnostic features, species-specific identification on the basis of microscopic morphology and reproductive structures (e.g., conidiophores, conidia, and conidial ontogeny) is often difficult. This is further exacerbated by the fact that many meristematic fungal species are highly pleomorphic, with anamorph life cycles and widely divergent methods of propagation. Some species display meristematic growth as the only type of reproduction, consisting of isodiametrically dividing cells and endoconidiation, which do not allow delimitation of taxa [11]. Thus, morphology gives only a presumptive identification at genus level, and the use of physiological characteristics (e.g., nitrogen and carbohydrate assimilation tests, range of growth at different temperature and proteolytic activity) is helpful for their identification.

Botryomyces caespitosus may be detected from surfaces by tape lifts or tease mounts from bulk samples. The laboratory diagnosis of chromoblastomycosis is performed by the demonstration of sclerotic bodies upon direct microscopical examination of wet KOH mounts of aspirated pus, skin scrapings, or biopsy material. However, *B. caespitosus* causes, and only occasionally, a chromoblastomycosis-like infection; it is in fact a phaeohyphomycosis.

Colonies on malt extract agar are restricted, cauliflower-like, heaped, pale brown initially becoming brown-black with age. Microscopy reveals that hyphae and budding cells are absent. The thallus is composed of clumps of irregularly septate, thick-walled cells, which are subhyaline, becoming dark brown with age. These disarticulate into smaller cell packets. Blastic conidia are in fact occasionally present. A series of approximately 50 physiological tests (e.g., growth on glucose, arabinose, salicin) are also available [3]. Hence, some useful characters are present in the case of this species at least. *B. caespitosus* differs from *Sarcinomyces phaeomuriformis* by young colonies being pink.

Molecular techniques have been applied for the identification of meristematic black fungi including *B. caespitosus*. Polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analyses of amplified small subunit ribosomal gene (SSU, 18S rRNA) and large subunit ribosomal gene (LSU, 5.8S rRNA and interspacers ITS1, ITS2) are employed [4,11–14].

5.2 METHODS

5.2.1 Sample Preparation

Molds from a specimen are grown on inhibitory mold agar, modified Sabouraud agars, or potato dextrose agar. Microscopic structures are observed on tease or tape preparations and slide cultures for up to 21 days.

After growth for 1–7 days on potato dextrose agar slants, lysates are prepared from approximately 1 cm^2 of mycelia with IDI lysis kits (GeneOhm Sciences, San Diego, CA). Briefly, in a biological safety cabinet, mycelia are collected by scraping the slant with a sterile stick in 1 mL of sterile, molecular-grade H_2O. The material is transferred to a 2 mL screw-cap tube. The tubes are centrifuged for 1 min at 6000 × *g*. If the mycelia does not pellet, the material is contained with a pediatric blood serum filter (Porex Corp., Fairburn, GA). The supernatant is removed. The material is resuspended in 200 µL of IDI sample buffer and transferred to the lysis tube, which contains glass beads. Lysis tubes are vortexed on the highest setting for 5 min. The tubes are placed in a boiling water bath for 15 min and centrifuged for 5 min at 16,000 × *g*. The supernatant is stored at −20°C until amplification [13].

5.2.2 Detection Procedures

Pounder et al. [13] described a real-time PCR with SYBR green DNA-binding dye and amplicon melting temperature analysis for fungal detection using pan-fungal primers ITS1 forward (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 reverse (5′-TCCTCCGCTTATTGATATGC-3′) that cover the internal transcribed spacer 1 (ITS1)–5.8S–ITS2 rRNA gene cluster (ITS). The identity of the fungi is verified by subsequent sequencing analysis.

Procedure

1. PCR mixture is composed of 1 × Lightcycler FastStart DNA Master Hybridization Probes mixture (Roche Applied Science) (containing deoxynucleoside triphosphates, FastStart *Taq* DNA polymerase, and 1 mM $MgCl_2$, additional $MgCl_2$ is added to a final concentration of 4.6 mM), 0.4 µM each of ITS1 forward and ITS4 reverse primers, 1 × SYBR green (Molecular Probes), and 3 µL template DNA.

2. Thermal cycling parameters include 95°C for 10 min; 50 cycles of 95°C for 5 s, 60°C for 20 s, and 76°C for 30 s; and a final extension at 72°C for 2 min.
3. The quality of the amplicon is determined using the derivative of the melt analysis curve (55°C–99°C, 45 s hold at 55°C, 5 s/°C) using the RotorGene 3000 (Corbett Robotics, Inc).
4. The amplified product is purified for bidirectional sequencing using ExoSAP-IT (USB Corp). Five microliters of Big Dye Terminator Ready Reaction Mix v. 1.1 (Applied Biosystems) is added to 4 µL of each primer (0.8 pmol/µL) and 3 µL of purified PCR product. Cycle sequencing is performed with a 9700 thermal cycler (ABI), using 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing reaction products are passed through a Sephadex G-50 fine column to remove unincorporated dye terminators. Purified sequencing reaction products are run on an ABI Prism 3100 Genetic Analyzer with a 50 cm capillary array.
5. Sequences are analyzed with the SmartGene Integrated Database Network software version 3.2.3 vr. SmartGene is a web-based software and database system with reference sequences derived from the National Center for Biological Information (NCBI) GenBank repository.

Note. Sequence-based identifications are defined by percent identity: species, ≥99%; genus, 93%–99%; and inconclusive, ≤93%. For strains producing discrepant identification between the methods based on phenotypic characteristics and ITS sequence analysis, the D1–D2 region of the large-subunit RNA gene is amplified with primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) and sequenced for species clarification [15].

5.3 CONCLUSION

Botryomyces caespitosus is a black fungus in the mitosporic Dothideomycetes group that is responsible for a phaeohyphomycosis in humans. The organism is also associated with biodeterioration of monuments. Given the close morphological similarity among black fungi, use of molecular techniques such as PCR and sequencing is critical for accurate and specific identification of the fungus.

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AUTHOR QUERIES

[AQ1] Please check the sentence beginning “Clinical symptoms may…” for completeness.

[AQ2] Please provide accessed date for reference [1].